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Co-digestion of cow and sheep manure: Performance evaluation and relative microbial activity

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ABSTRACT

This study evaluated the performance of anaerobic co-digestion of cow manure (CM) and sheep manure (SM) in both batch and continuous digesters at 37 °C. Synergistic effects of co-digesting CM and SM at varying volatile solids (VS) ratios (1:0, 0:1, 3:1, 1:1, 1:3) were observed in the batch experiment, with the most effective degradation of cellulose (56%) and hemicellulose (55%), and thus, the highest cumulative methane yield (210 mL/gVS$_{added}$) obtained at a CM:SM ratio of 1:3. Co-digesting CM and SM improved the hydrolysis, as evidenced by the cellulase brought by SM and the increases of cellulolytic bacteria *Clostridium*. Besides, co-digestion enhanced the acidogenesis and methanogenesis, reflected by the enrichment of syntrophic bacteria *Candidatus Cloacimonas* and hydrogenotrophic archaea *Methanoculleus* (Coenzyme-B sulfoethylthiotransferase). When testing continuous digestion, the methane yield increased from 146 mL/gVS/d (CM alone) to 179 mL/gVS/d (CM:SM at 1:1) at a constant organic loading rate (OLR) of 1g VS/L/d and a hydraulic retention time (HRT) of 25 days. Furthermore, the anaerobic digestion process was enhanced when the daily feed changed back to CM alone, reflected by the improved daily methane yield (159 mL/VS/d). These results provided insights into the improvement of methane production during the anaerobic digestion of animal manure.

1. Introduction

Intensive livestock farming has been increasingly developed over the last decades to satisfy our overwhelming demand for meat and dairy products [9]. However, these farms inevitably contribute to a considerable amount of manure annually [6]. Disposal of untreated manure not only causes serious environmental problems such as soil contamination, eutrophication, and greenhouse gas emissions but also fails to make full use of the nutrients in it. Recently, many anaerobic digestion (AD) systems are being operated on those farms for the simultaneous waste treatment and production of biogas, with cow manure (CM) frequently used due to its high availability [10,21,34,38]. However, mono-digestion of CM inherently delivers low biogas yield because the cow-feed (mostly grass) has already been processed by the microorganisms in cow's rumen, leaving CM with high lignocellulosic contents (cellulose, hemicellulose and lignin) which is relatively resistant to AD [44,47]. As a result, the hydrolysis of CM in AD is rather slow, making it the rate-limiting step [15,26]. The difficulty can be partly overcome by a pre-treatment which breaks up the complex structure (mainly between hemicellulose and lignin) and makes the lignocellulosic compounds more accessible for hydrolytic microbes. Another approach is through anaerobic co-digestion to increase the number of indigenous microorganisms with efficient degradation capabilities [35]. Both solutions are well-established with the co-digestion preferable due to its low energy input [22,35]. As illustrated by Amon et al. [1], a negative correlation between lignin content and final methane yield was obtained using CM as the substrate. To avoid lignin inhibition, material with a low lignin content is preferred to co-digest with CM to generate maximum methane yield [1]. Moreover, one contains active specialized hydrolytic microorganisms that work on the depolymerization of lignocellulose is also preferred as a co-digestion partner.

Unlike CM, sheep manure (SM) is rarely mentioned as an addition in AD despite its recently proven ability for high methane yield [20,48]. This is surprising, as the number of sheep ranks the first among the total livestock population worldwide, making SM a non-neglectable source of manure waste [10,37]. Besides, SM

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possesses lower lignin compounds [17,20,24]. Another intriguing point of SM is that it may contain hydrolytic microbes that could boost the hydrolysis step in AD [14], but the species of these microbes remained undocumented. Hence, it hints that SM is more than a co-digestion substrate, but also harbors active microbes enhancing the hydrolysis of recalcitrant substrates. Recently, Martí-Herrero et al. [24] used tubular reactors fed with CM or with a mixture of CM and SM at psychrophilic conditions (16.6 °C) to investigate the bioprocess efficiency. They found that a 4:1 ratio of CM and SM generated almost twice as much methane as when using CM alone. Cestonaro et al. [7] used sheep bedding and CM as the substrate in a batch AD process at room temperature (18.6 °C) and found that co-digestion of sheep bedding and ≥50% cow manure increased biogas production by only 3.4%–4.3% compared with CM alone.

Although the ambient temperature can be applied in specific areas as indicated by the aforementioned research, mesophilic conditions (25C-40 °C) are still suggested in the agricultural sector to fulfil the biogas potential of manure [42]. Detailed knowledge on the influence of adding SM to CM, and thus, the effect on the methane production, remains unknown. Therefore, this study thoroughly investigated the AD performance of co-digestion of CM and SM in both batch and continuous assays. Varying CM:SM co-digestion ratios (1:0,3:1,1:1,1:3,0:1, on volatile solids basis) were tested in batch trials to assess their synergistic effects. Microbial tools were performed to unveil the core microbes responsible for the improved methane yield. Such synergy was further demonstrated in the continuous system.

2. Materials and methods

2.1. Feedstock and inoculum

The CM and SM were taken from a sheep and cow dairy farm nearby Groningen, the Netherlands. The inoculum was taken from an anaerobic digester fed with aerobic sludge from a municipal wastewater treatment plant (WWTP, Garmerwolde, Groningen). After collection, all substrates and inoculum were kept at 4 °C in a cold room. Before use, the inoculum was acclimated in an incubator at 37 °C for 10 days to reduce the residual methane production from the inoculum in AD. Key physicochemical characteristics of CM, SM, and inoculum are summarized in Table 1.

2.2. Batch digester start-up and experimental design

The batch AD of CM and SM was carried out at five CM/SM ratios based on volatile solids (VS): 1:0, 0:1, 1:1, 3:1, 1:3. The experiments were performed in 500 mL glass bottles capped with butyl rubber stoppers with a working volume of 400 mL. For each treatment, an initial substrate concentration of 25 gVS/L was used. The substrate to inoculum ratio (ISR) was set 0.5 [13]. After adding the proper amounts of substrate and inoculum, each bottle was filled with distilled water to achieve the working volume. Sulfuric acid was used to adjust the initial pH to 7. The bottles were then flushed with pure nitrogen gas for 5 min to ensure anaerobic conditions. Finally, the bottles were placed in a shaking incubator (140 rpm) at 37 ± 1 °C. Each experiment was done in triplicate, and bottles with inoculum alone were used as a control. Samples for biogas composition analysis were obtained from the headspace of the bottles. Biogas and methane yields from the control were subtracted from the data obtained in the experiments with CM and/or SM. Biogas production and methane content were measured daily.

Table 1
Characteristics of cow manure (CM), sheep manure (SM), and inoculum.

Characteristics	CM	SM	Inoculum
TS (% FM)	19.99 (0.28)	22.27 (0.02)	5.14 (0.04)
VS (% FM)	16.95 (0.15)	18.69 (0.01)	3.24 (0.04)
VS/TS	84.78 (0.59)	83.73 (0.34)	63.09 (0.34)
pH	8.59	8.07	7.60
TCOD (g/kg FM)	35.50 (2.12)	62.91 (0.07)	6.75 (0.11)
SCOD (g/kg FM)	22.30 (0.35)	45.97 (2.75)	3.69 (0.44)
Cellulose (% TS)	15.31 (0.61)	11.63 (0.68)	ND
Hemi-cellulose (% TS)	14.05 (0.34)	13.27 (0.69)	ND
Acid soluble lignin (% TS)	1.06 (0.07)	1.22 (0.08)	ND
Acid insoluble lignin (% TS)	12.91 (0.89)	7.37 (0.39)	ND
Extractive (% TS)	47.18 (0.46)	61.97 (0.99)	60.55 (0.44)
Ash (% TS)	15.59 (0.15)	16.08 (0.05)	27.69 (0.33)
C (% TS)	42.11 (0.22)	42.40 (0.41)	30.23 (0.22)
N (% TS)	2.23 (0.03)	2.91 (0.06)	4.59 (0.04)
H (% TS)	5.91 (0.01)	6.13 (0.04)	5.08 (0.03)
O (% TS)	33.76 (0.31)	32.09 (0.45)	21.97 (0.68)
S (% TS)	0.39 (0.05)	0.38 (0.02)	1.22 (0.05)
C/N	18.85	14.59	6.58

The values are means (n = 3) with the standard deviation between brackets. ND=Not detected.

The cumulative methane yield (CMY) of each treatment was calculated by dividing the cumulative volume of methane produced after complete anaerobic degradation by the mass of initial VS of substrate added. The experiment ended when there was no further gas detected. At the beginning and the end of the experiment, samples were taken and stored at −20 °C for chemical and compositional analysis, and 5 mL samples were mixed with 5 mL ethanol (100%) and stored at −20 °C for further microbial population analysis.

2.3. Continuous digester set up and design

A single stage laboratory-scale continuously stirred tank reactor (CSTR) with a working volume of 2.4 L was used. The reactor was fed once a day by a peristaltic pump. An equivalent volume of digester content was discharged before feeding. The hydrolytic retention time (HRT) was set to 25 days based on the batch experiment, and the organic loading rate (OLR) was 0.5 g VS $L^{-1}d^{-1}$ from day 0 to day 50 and then increased to 1g VS $L^{-1}d^{-1}$. The temperature was maintained at 37 °C±1 °C by a water bath. A constant stirring speed of 120 rpm was used. The reactor started with 2.4L same seed sludge coming from WWTP as used in the batch experiments, followed by feeding once per day with CM alone or CM and SM. Biogas and methane content were measured daily and chemical analysis was conducted every 3 days in each phase. The detailed experimental design and information of the substrate used for the continuous test are shown in Table S1, Supplementary data.

2.4. Biogas measurement and mass removal calculation

The volume of daily biogas production was determined by displacing a 75% saturated solution of sodium chloride, which was acidified to pH 2.0 [30]. Biogas composition was analyzed by gas chromatography (C2V-200 Micro GC, Thermo Scientific) with a GCC200-U-BND cartridge and a thermal conductivity detector (TCD). The temperature of the column, injector, and detector was 60 °C, 120 °C, and 120 °C, respectively. Helium was used as carrier gas. The measured biogas and methane volumes were then adjusted to the volumes of dry gas at standard temperature (0 °C)

and pressure (1 atm) [15].

The theoretical methane yield (TMY) of CM and SM was calculated based on Li et al. [20]. The biodegradability is defined as the ratio of CMY and TMY. For different co-digestion treatments, the simulated methane yields of different mixtures were calculated based on the proportion of CM and SM in the mixtures as well as methane yields coming from CM and SM alone in AD as shown in Eq. (1) [49].

$$M_{mixture,i} = M_{CM,i} \times Y_1\% + M_{SM,i} \times Y_2\% \quad (1)$$

where i = the duration of AD (d), $M_{mixture,i}$ = the simulated methane yield of mixture at the ith day (mL/g VS_{added}), $M_{CM,i}$ = methane yield of CM at the ith day, $Y_1\%$ = the percentage of CM in the mixture (%), $M_{SM,i}$ = methane yield of SM at the ith day, and $Y_2\%$ = the percentage of SM in the mixture (%).

The TS or VS removal rate in the batch test was calculated from the total mass removal from the reactors and blank reactors as shown in Eq. (2) [16].

$$S_R(\%) = (F + I) \times a - I \times b / F \quad (2)$$

where S_R = TS or VS removal of substrate (%), F = total TS or VS added to the reactor (g), I = total TS or VS inoculum added to the reactor (g), a = calculated TS or VS removal of feed plus inoculum based on total initial and final mass of TS or VS present in the reactor (%), b = calculated TS or VS removal of inoculum in blank reactor (%).

2.5. Analytical methods

TS% and VS% were determined following the standard methods [2]. The pH was measured with a digital pH meter (H160, Hach, Germany). Soluble fractions of all the samples were obtained by centrifuging at 10,000 rpm for 10 min at 4 °C. The supernatant was filtered through a cellulose acetate membrane with a pore size of 0.45 μm to obtain the soluble fraction. The soluble fraction was used to determine the soluble chemical oxygen demand (SCOD), and total ammonia (TAN). Total soluble oxygen demand (TCOD), and SCOD were measured with specific kits (LCK 014, Hach, Germany). TAN was measured with a rapid ammonia assay kit (K-AMIAR, Megazyme, USA). Free ammonia (NH_3–N) was determined according to the equation based on both pH and temperature as described elsewhere [11]. Total volatile fatty acids (TVFAs) and total alkalinity (TA) were analyzed by titration with 0.1 N H_2SO_4 to the endpoints of pH 5.0 and 4.4 with an auto-titrator (AT1000, Hach, Germany) [17]. Air-dried CM, SM, and inoculum samples were used for elemental analysis with an elemental analyzer (Vario EL/micro cube, Germany).

CM and SM were pre-treated following the method based on the National Renewable Energy Laboratory (NREL) Analytical Procedure. Extractive-free samples were subsequently used to determine the structural carbohydrates and lignin with a two-step acid hydrolysis method [36]. Monomeric sugars and cellobiose were analyzed with high-performance liquid chromatography (HPLC) (Agilent Technologies 1200 series) equipped with a Bio-Rad Aminex HPX-87H 300 × 7.8 mm column at 60 °C using 5 mM H_2SO_4 as eluent. Then, the exact amount of cellulose and hemicellulose was calculated from the monomeric sugars concentrations based on NREL: 'Determination of Structural Carbohydrates and Lignin in Biomass' [36].

2.6. High-throughput 16s rRNA gene sequencing and analysis

Samples were fixed by adding ethanol to a final concentration of 50% (v/v) pending DNA and RNA extractions. The fixed samples were sent for sequence analysis using the BioProphyler approach (Bioclear Microbial Analysis BV, Groningen, Netherlands). Briefly, total RNA was extracted using the FastRNA ProSoil Direct kit (MP Biomedicals, Solon, OH, USA) and cDNA was synthesized using the iScript™ Reverse Transcription Supermix with random primers (Biorad, Hercules, USA). High-throughput sequencing was performed on an Illumina MiSeq. The reads, maximal 301 bp in size, were processed using in-house developed BioProphyler software. The sequence reads were first run through a quality filter named Trimmomatic [5], followed by a comparison to sequences stored in GenBank using the BLASTn algorithm (http://www.ncbi.nlm.nih.gov/BLAST) and the RAPSEARCH2 algorithm (https://doi.org/10.1093/bioinformatics/btr595). Identifications were ranked by tag abundance. Relative abundance (RA) was calculated from the number of sequences that affiliated with a taxon divided by the total number of sequences per sample. Additionally, Alpha diversity metrics were calculated for each sample for Shannon and Simpson indices, Beta diversity was calculated with the weighted UniFrac distance matrix and then visualized using principal coordinates analysis (PCoA). Furthermore, canonical correspondence analysis (CCA) was performed using the R package to examine correlations of bacterial and archaeal genera with environmental factors and reactor performance with pH (7.45–7.58), TAN (1996–2211 mg/L), CMY (157–210 mL/g), and cellulose removal (38%–56%). RA of the 19 major bacterial genera (>1%) and 6 major archaeal genera (>1%) detected were used in the CCA analysis.

2.7. Data analysis

Differences in the methane yields obtained in co-digestion of CM and SM at different ratios in both batch and continuous experiments were evaluated by using single-factor analysis of variances (ANOVA) in Excel 2013. Statistical significance was tested using Student's t-test with a threshold p-value of 0.05.

3. Results and discussion

3.1. Substrate characterization

Noticeably, SM contained significantly lower lignin content (8.59%) than CM (13.97%) ($p<0.05$). In the elemental analysis, CM and SM could be represented as $C_{21.99}H_{37.04}O_{13.26}N_1$ and $C_{17.02}H_{29.51}O_{9.66}N_1$, respectively. Hence, the corresponding TMYs for CM and SM were 357 mL/gVS_{added} and 395 mL/gVS_{added}, respectively. These findings suggested that the co-digestion of CM and SM might have a better performance than the mono-digestion of CM (Table 1).

3.2. Methane production of CM and SM

Methane production started immediately from the first day for all digestion tests (Fig. 1). For CM alone, one peak was observed which occurred on day 1 (11 mL/g VS-d), the methane production rate remained relatively constant from day 2 to day 4 and then declined gradually. For SM alone, the peak occurred on day 2 (16 mL/g VS-d) and from day 3 to day 7, the methane production rate remained stable, with little fluctuation, and then decreased gradually. The higher peak value observed in SM than in CM could be attributed to the higher concentration of TVFAs observed in SM than in CM at the beginning of the experiment, as they can be quickly converted into methane via acetogenesis and methanogenesis (Table 2).

Eventually, the CMY obtained from SM (207 mL/gVS_{added}) was 1.3-fold higher than that from CM (157 mL/gVS_{added}). The methane

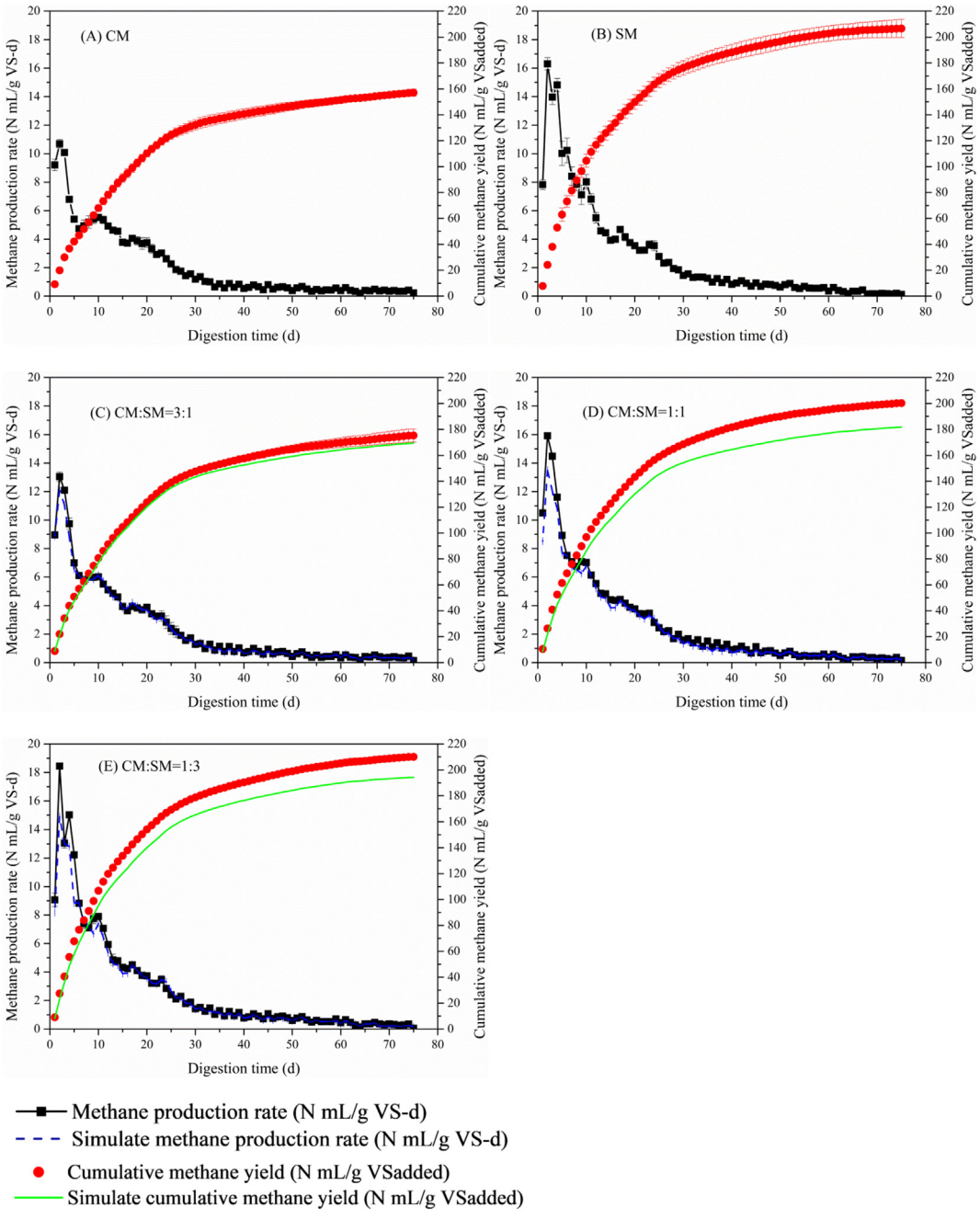


Fig. 1. Methane production rate and methane cumulative yield for co-digestion of cow manure (CM) with sheep manure (SM) at mixing ratio of 1:0, 0:1, 3:1, 1:1,and 1:3 based on VS. The values are means (n = 3).

yield of CM in this experiment was similar to the yield found by Vivekanand et al. [39], and it also fell in the range of 125–166 mL/gVS_{added} reported by Amon et al. [1]. Compared with CM, SM is slightly easier biodegradable, presumably because of its lower lignin content and a higher amount of extractives (acids and oligomers) (Table 2). The methane yield observed in this experiment was similar with the methane yield (204 mL/gVS_{added}) reported by Riggio et al. [29] and slightly higher than the methane yield (188 mL/gVS_{added}) obtained by Cestonaro et al. [7]. Also, the biodegradability of CM (44%) was significantly lower than that of SM (52%), which was following the CMYs observed for mono-digestion of CM and SM ($p<0.05$).

3.3. Methane production from the anaerobic co-digestion of CM and SM

For the co-digestion of CM and SM at mixing ratios of 3:1, 1:1, and 1:3, there was one peak of daily methane yield observed at day 2, which was 13 mL/gVS-d, 16 mL/gVS-d, and 18 mL/gVS-d, respectively. Compared with the simulated co-digestion of CM and SM, the peak values of daily methane yield were enhanced by 7.8%, 17.9%, and 23.8%, respectively for the different mixing ratios of CM and SM. Such enhancement may be ascribed to more available fermentable compounds (sugars and acids) brought by the co-digestion than the mono-digestion. Another possible explanation could be the co-digestion promotes the activity of microbes,

Table 2
Chemical parameters of different digestates.

Chemical parameters		CM	CM:SM = 3:1	CM:SM = 1:1	CM:SM = 1:3	SM
pH	Initial	7.00	7.00	7.00	7.00	7.00
	Final	7.47(0.01)	7.45(0.02)	7.51(0.01)	7.58(0.02)	7.55(0.04)
TVFAs (mg $CaCO_3$/L)	Initial	1351(34)	1466(7)	1491(19)	1590(13)	1654(38)
	Final	756(1)	767(34)	801(11)	824(6)	846(11)
TA (mg CH_3COOH/L)	Initial	2953(48)	2844(40)	2837(22)	2766(18)	2738(47)
	Final	4471(56)	4595(75)	5031(25)	5013(50)	5115(69)
TVFAs/TA	Initial	0.415(0.007)	0.515(0.01)	0.521(0.008)	0.575(0.005)	0.607(0.006)
	Final	0.169(0.003)	0.167(0.005)	0.167(0.002)	0.164(0.003)	0.165(0.001)
TAN (mg/L)	Initial	1663(80)	1487 (21)	1498(28)	1576(26)	1554(72)
	Final	2211(90)	2030(63)	2207(53)	1996(106)	2177(43)
FAN (mg/L)	Initial	21(1)	19(0)	19(0)	20(0)	20(1)
	Final	81(3)	71(2)	88(2)	93(5)	95(2)
TCOD (mg/L)	Initial	13435(101)	13045(75)	12995(80)	13755(27)	12935(94)
	Final	4989(80)	5450(180)	6852(51)	6482(132)	6958.66(211)
SCOD (mg/L)	Initial	4202(177)	4852(76)	4630(93)	4853(152)	4786(74)
	Final	2633(152)	2888(106)	3035(154)	3270(58)	3041(32)

The values are means (n = 3) with the standard deviation between brackets.

especially the activity of methanogens.

The CMYs of co-digestion of CM and SM at mixing ratios of 3:1, 1:1, and 1:3 were 175, 200, and 210 mL/gVS_{added}, respectively (Fig. 1), which showed a higher methane yield of 11.6%–30.5% than mono-digestion of CM. Compared to the simulated CMYs of different mixing ratios of CM and SM, the actual CMYs of co-digestion of CM and SM at mixing ratios of 3:1, 1:1, and 1:3 increased by 3.4%, 10.1%, and 8.2%, respectively (Fig. 1), indicating that a synergistic effect took place during co-digestion of CM and SM. A similar phenomenon was observed by researchers who compared co-digestion of CM and chicken manure with mono-digestion of CM or chicken manure alone in AD, strengthening the hypothesis that co-digestion of CM and other types of lignin-poor manure could boost the methane production compared with mono-digestion of CM alone [43].

3.4. Characteristics of the digestates

To further examine the process, the characteristics of the digestates were measured on day 0 and day 75 regarding the chemical parameters listed in Table 2. The pH of the digestates in all reactors at day 75 (7.45–7.58) were all higher than the initial value of pH 7.0. Similarly, the TAN values increased among all treatments as a result of the gradual degradation of nitrogenous compounds in CM and SM. TAN plays a critical role in the stability of AD, especially for nitrogen-rich materials such as manure and sludge. The threshold concentration of TAN in AD is generally regarded as 2500 mg/L when treating cattle wastes [12]. At the end of the experiment, the highest value of TAN in the digestates at day 75 was 2211 mg/L of CM alone. Whereas, the co-digestion mixtures showed lower TAN values, ranging from 1996 mg/L to 2207 mg/L, with the lowest value of 1996 mg/L observed in co-digestion of CM and SM at ratio 1:3. Hence, co-digesting CM with SM may reduce the concentration of TAN to guarantee a stable AD environment [12]. As for the ratio of TVFAs and TA, it is recommended that a ratio of 0.3–0.4 or even less is the optimal range for a successful batch experiment while a ratio of 0.6 or more indicates an excessive biomass input [28]. Different from the unbalanced TVFAs/TA ratio in the co-digests than in CM alone on day 0, no significant differences of the TVFAs/TA ratio among all digesters were found when AD finished, which ranged from 0.164 to 0.169, strongly implying that the addition of SM didn't disturb the whole AD process ($p > 0.05$) (Table 2). The higher TVFAs removal obtained in the co-digestion experiments (46%–49%) than CM alone (44%) was also in line with higher CMYs, since TVFAs are major intermediates in AD that are converted into methane.

3.5. Degradation of organic components

The final methane yield in AD is dependant on the degradation efficiency of organic compounds. The removal of TS, VS, and organic components from the AD experiments with different ratios of CM and SM are shown in Fig. 2. Along with the methane profiles, the removal of VS for all mixtures (43–49%) and SM alone (52%) was significantly higher than for CM alone (37%) ($p<0.05$). Hence, co-digestion of CM and SM may contribute to an improved decomposition of solid fraction and 'untrap' more products for a further conversion into methane compared with CM alone.

Lignocellulose is the recalcitrant component of CM and SM but contributes largely to the methane yield. In this experiment, the removal of cellulose and hemicellulose in SM alone (56% and 54%) was significantly higher than that in CM (38% and 31%) ($p<0.05$). Similarly, a higher removal was obtained in the mixtures, ranging from 43% to 56% (cellulose) and 38%–55% (hemicellulose), respectively ($p<0.05$). This observation could be ascribed to the lower lignin content in SM than in CM, which could make

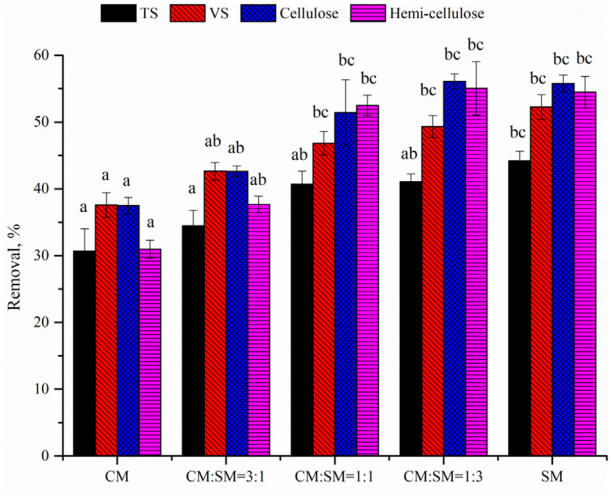


Fig. 2. TS, VS, cellulose, and hemicellulose removal rates for co-digestion of cow manure (CM) with sheep manure (SM) at mixing ratios of 1:0, 0:1, 3:1, 1:1, and 1:3. The values are means (n = 3). (*Note*: For components that have different letters, the removal is significantly different ($p < 0.05$)).

lignocellulose more accessible by lignocellulosic-degrading microbes. On the other hand, it could be attributed to the presence of more active hydrolytic bacteria in SM.

3.6. Microbial community analyses

3.6.1. Comparison of overall community diversity and similarity

The alpha diversity measurements of the samples were represented using operational taxonomic units (OTUs), Shannon and Simpson diversity index (Table S2). Both samples on day 0 showed higher values of these diversity measurements than the samples on day 75, indicating that the microbial diversity decreased throughout the AD process. Specifically, on day 75, both indexes were found lower in nearly all co-digestions than CM or SM alone except for CM:SM at 3:1. The indexes on day 75 of archaea were found higher, except for CM:SM at 3:1. The differences indicated that the co-digestion of CM and SM was more beneficial for archaea then for bacteria, especially in the presence of higher amounts of SM (≥50%).

Beta diversity was calculated and PCoA was performed to examine the similarity of the microbial communities among the samples (Fig. S1). To point out, on PC2, a gradual separation of the samples with different CM and SM ratios was shown. The samples with a high amount of SM (≥50%) and SM alone were separated from low amounts of SM and CM alone on day 75. Thus, the microbial community in CM-containing digesters was fairly constant unless more SM (≥50%) was added to co-digest with CM.

3.6.2. Bacterial community composition and dynamics

3.6.2.1. Bacterial community dynamics at the phylum level. The major bacteria phyla detected in both mono and co-digestion of CM and SM are shown in Fig. 3. Several phyla were found ubiquitous, with high share (more than 1%), and included *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Chloroflexi*, and *Cloacimonetes*. *Firmicutes* were the predominant phylum in all digests on day 75 and are known to contain members of cellulolytic bacteria and syntrophic bacteria, which can degrade various VFAs. Thus, the contribution of this phylum in our study was probably related to the degradation of cellulolytic materials into intermediate products such as VFAs and the further utilization of these products. This phylum was found slightly higher in co-digestion scenarios (48–49%) than in CM alone (46%). In contrast, co-digestion was unfavorable for *Proteobacteria*, reflected by its low proportion in co-digests (15–19%) than in CM alone (24%). Similar to *Firmicutes*, most of the Alpha-, Beta-, Gamma- and Delta-proteobacteria are known for the utilization of glucose, acetate, propionate, and butyrate [3]. Differences on the RA of these two phyla may result from the competition in the given environment (co-digestion or mono-digestion). Apparently, *Cloacimonetes* thrives in co-digests, especially with 50% and 75% SM addition (8% and 10%, respectively), instead of CM alone (4%) (Fig. 3). This phylum has the potential to ferment amino acids and may serve as the H_2 producer [37]. The RA of two phyla remained almost unchanged in both co-digests and mono-digests. Members of *Bacteroidetes* can produce various lytic enzymes and acetic acid via the degradation of organic compounds [31]. The phylum *Chloroflexi* is an important glucose-degrading group [3].

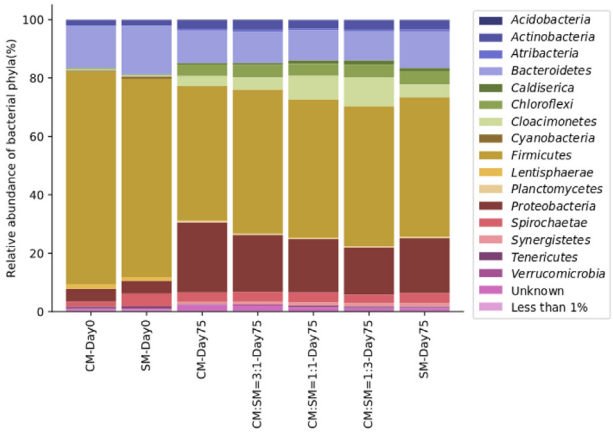


Fig. 3. Relative abundance of predominant bacterial phylum (more than 1.0% detected in at least one sample).

3.6.2.2. Bacterial community dynamics at the genus level. The distribution at the genus level is shown in Fig. 4. The genera *Clostridium*, *Ruminococcus*, *Ruminococcaceae*, and *Intestinimonas* were predominant within samples for CM and SM on day 0 but not on day 75 except for *Clostridium*. Both *Ruminococcus* and *Ruminococcaceae* were reported to have the potential to ferment cellulose, and they are present mainly within the animal gut [8]. Similarly, some *Clostridium* species can hydrolyze cellulose in AD [18]. Together with *Clostridium*, the profusion of *Romboutisia* and *Turicibacter* that belong to *Firmicutes* were observed in all digests. They were found to be involved in carbohydrate metabolism [33]. Compared with CM alone, higher proportions of these two genera were observed, especially when ≥50% of SM was added (Fig. 4). Similarly, *Clostridium* showed an increasing proportion with the gradual addition of SM, increasing from 7% in CM alone to 10% in 75% addition of SM. This was also in line with previous findings that a higher proportion of *Clostridium* correlated with a better performance in the manure-based AD [46]. Furthermore, the appearance of genera *Smithella*, *Syntrophus*, and *Delftia* that belong to the phylum *Proteobacteria* might also indicate their roles in acetogenesis in our test. The genus *Smithella* could conduct propionate oxidation, whereas *Syntrophus* species are involved in butyrate oxidation [8]. No apparent change was observed in co-digests and mono-digest with those two genera. It is noteworthy that a high proportion of the genus *Candidatus Cloacimonas* was observed only in digesters with a high amount of SM, it reached 8% and 10% in 50% and 75% addition of SM, respectively. As uncovered by Pelletier et al. [27], this genus could obtain most of its energy from fermenting amino acids and sugars to generate propionate, CO_2, and H_2. It is further identified as a syntrophic bacterium, associated with hydrogenotrophic methanogen to generate methane [37,45]. The importance of *Candidatus Cloacimonas* in acidogenesis and its syntrophic ability was also illustrated by Li et al. [21] who used dairy manure in a farm-scale plug-flow loop reactor. Thus, the prosperity of *Candidatus Cloacimonas* in the co-digests may contribute an improved methane yield (Fig. 1).

3.6.3. Archaeal community dynamics at the genus level

The archaea community at day 0 and day 75 is shown in Fig. 5. The predominant archaea shifted from *Methanocorpusculum* and *Methanosaeta* on day 0 to *Methanosaeta*, *Methanospirillum* and *Methanoculleus* on day 75, respectively. Specifically, the acetotrophic genus *Methanosaeta* was dominant within all digests. It may hold its competitive advantage under low TVFAs (especially low acetate) concentration due to its strong affinity with acetate [23,32]. As the compensation metabolism in our study, the hydrogenotrophic genera *Methanospirillum*, and *Methanoculleus* accounted for 20%–26% in different digests, with varying share. However, these archaeal guilds can't be overlooked, especially *Methanoculleus*, as this genus was previously found dominant in the batch AD treating cellulose-rich, lipid-rich, and protein-rich substrates [40]. It was noteworthy that in our study, higher proportions of *Methanoculleus* were detected in the co-digestion of CM with ≥50% of SM (14%–16%) than

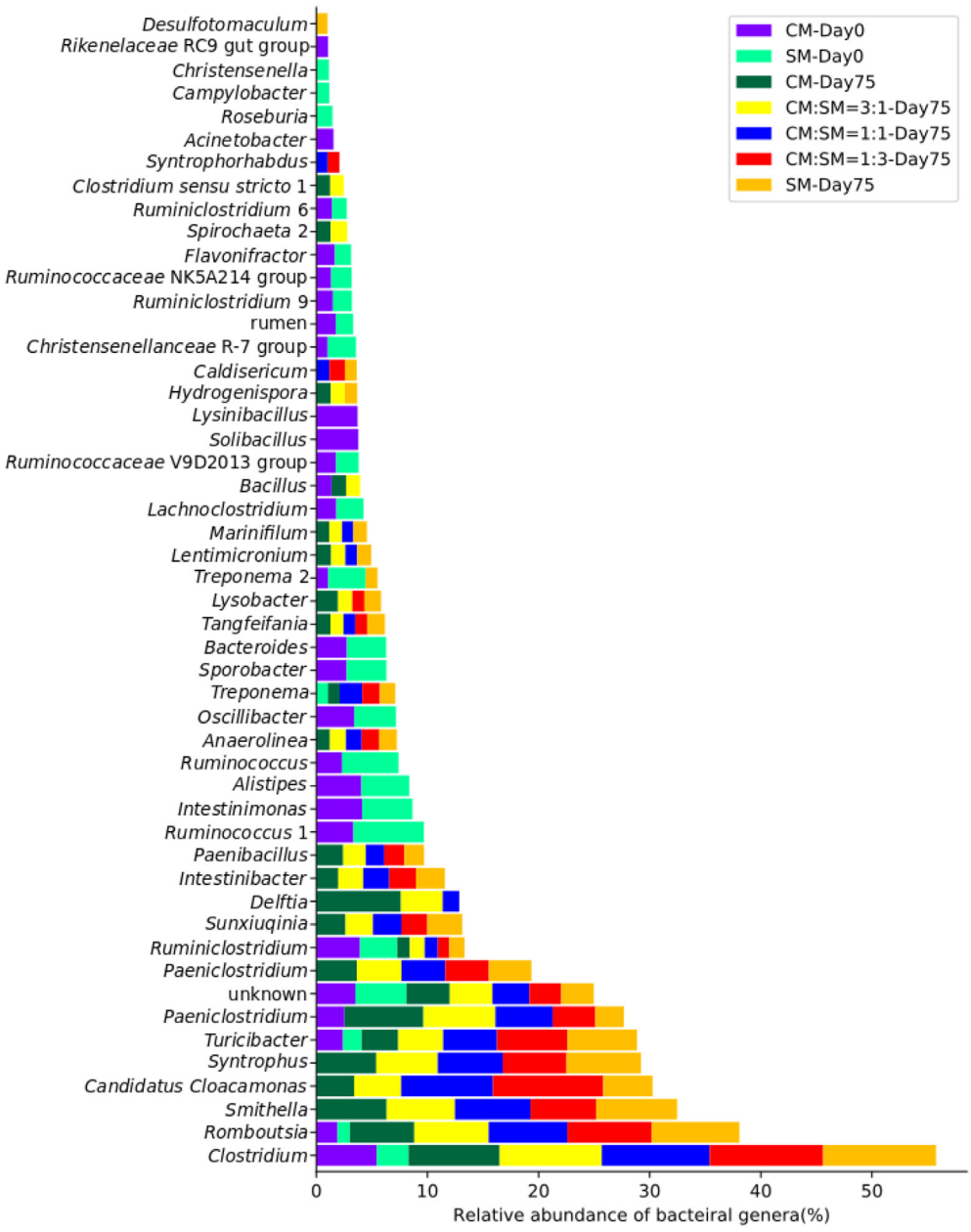


Fig. 4. Relative abundance of bacterial genera (more than 1% detected in at least one sample).

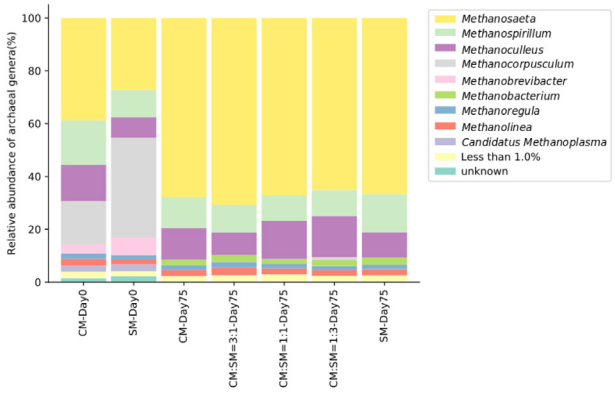


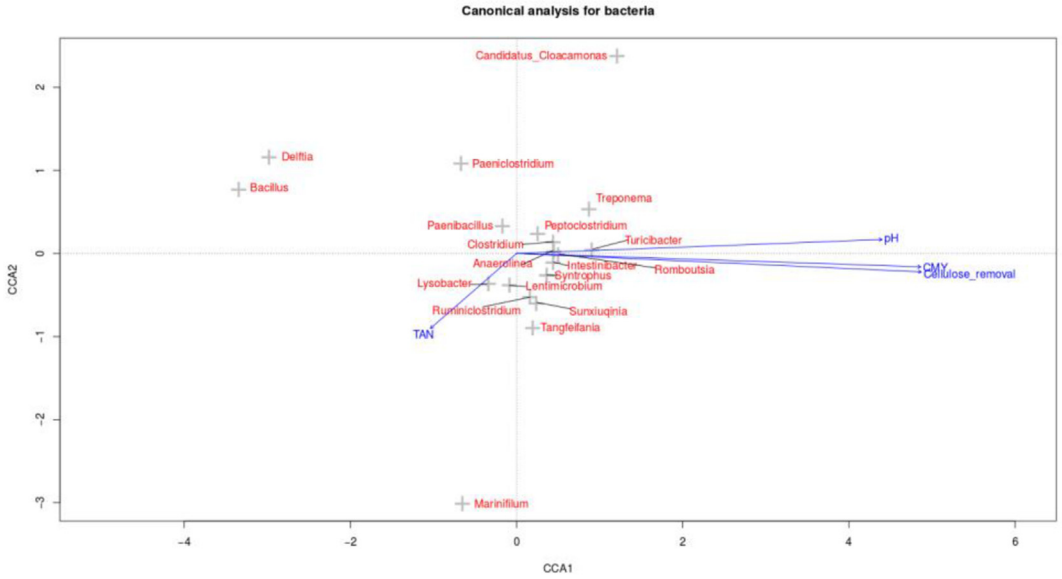
Fig. 5. Relative abundance of archaeal genera (more than 1% detected in at least one sample, for the methanogens below 1%, see supplementary Table S4).

CM (10%) or SM (8%) alone (Fig. 5). The increase of *Methanoculleus* might be associated with the increase of members of the genus *Candidatus Cloacimonas* as H_2 producers from the fermentation of (monomers of) lignocellulose. As a result, this microorganism might indicate a promoted lignocellulose removal in digesters with a high amount of SM and partially be the reason for the observed synergistic effect. Moreover, genera *Methanolinea*, *Methanoregula*, and *Methanobacterium* were found in a relatively low abundance (2–3%) in our experiments, indicating their limited role in methane formation. *Methanolinea* is normally found in sludge-based bioreactors [25]. *Methanoregula* and *Methanobacterium* are strictly hydrogenotrophic methanogens using H_2 and CO_2 to synthesize methane [41].

3.6.4. Correlation of microbial profiles with environmental factors

The environmental variables were found to have different

(a)



(b)

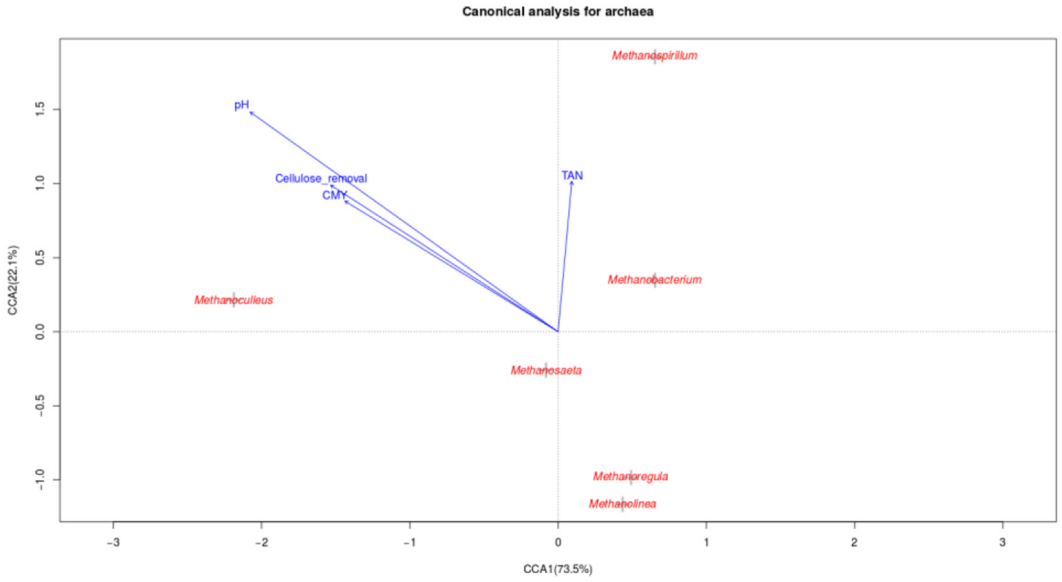


Fig. 6. Canonical analysis (CCA) for Bacteria (a) and Archaea (b) in relation to the environmental parameters pH, cumulative methane yield (CMY), cellulose removal, and total ammonia (TAN).

effects on shaping the bacterial and archaeal communities. In Fig. 6 (a), CMY, cellulose removal, and pH were positively intercorrelated, and all of them were negatively correlated with TAN. The bacterial genera such as *Clostridium*, *Candidatus Cloacimonas*, *Romboutisia* and *Turicibacter* appeared to be closely positively related to CMY, cellulose removal, and pH variables, while negatively related to TAN. The results further implied the contribution of these bacteria in carbohydrate metabolism, methane production and TAN reduction when treating manure waste, suggesting the merits of co-digestion CM and SM compared to CM alone.

The CCA results showed a distinctive correlation between environment variables and archaea genera, indicating their functional role in shaping the community of archaea (Fig. 6 (b)). *Methanoculleus*, *Methanospirillum*, and *Methanobacterium* were positively correlated with the environmental variables. *Methanosaeta*, *Methanolinea* and *Methanoregula* were negatively correlated instead. Since pH, CMY, and cellulose removal contributed most to CCA1 (73.5%), *Methanoculleus* was hence most shaped by these variables and was an indicator of good performance while using manure as the substrate in AD.

3.6.5. Functional enzymes analysis

The RNA sequence analysis revealed the expression of genes coding for active key-enzymes in the AD of CM and SM as well as their blends (Table 3 and Fig. S1). Enzymes involved in carbohydrate metabolism and methane metabolism are discussed here as they are major concerns in this study.

Enzymes such as cellulase, glycogen phosphorylase and alpha-amylase are known for hydrolysing polysaccharides. Compared with CM, SM contained more different bacteria that excrete these enzymes (Table 3). Particularly, *Bacteroides* sp. and an uncultured bacteria species were observed uniquely in SM that excrete cellulase, suggesting that SM may possess enzymes which could effectively degrade lignocellulosic compounds in CM. As the final step for methane production, enzymes involved in methane synthesis are also listed in Table 3. Coenzyme-B sulfoethylthiotransferase, also known as methyl-coenzyme M reductase (MCR), holds its predominance in methane formation in our study (>90%). It is responsible for combining the methyl group of coenzyme M with hydrogen from the coenzyme B to synthesize methane [4] (Fig. S2). MCR synthesized by *Methanothrix soehngenii* (*Methanosaeta*) accounted for more than 50% in all digesters. The high amount of MCR was in agreement with high RA of *Methanosaeta* in all cases (Fig. 5). Together with the archaeal profile, MCR synthesized by *Methanoculleus* sp. was found more active in CM and SM blends than CM alone, especially in reactors with a high amount of SM (CM:SM = 1:1 and 1:3), reaching 31% and 29% compared with CM (21%) (Table 3 and Fig. S2). Besides, Tetrahydromethanopterin S-methyltransferase and methylenetetrahydromethanopterin dehydrogenase (both for CO_2 and CH_4 formation) were identified in CM:SM at 1:1 and 1:3, backing up the fact that co-digestion could enrich the methane metabolism pathway, especially in reactors with a high amount of SM.

3.7. Continuous digester analysis

The batch test results implied the possibility of using SM to co-digest with CM at long-time run. Since comparable results were obtained with the CM:SM ratios at 1:1 and 1:3, the ratio 1:1 was used here. Based on the batch test results, the time needed to reach 80% of the CMY for CM was 25 days (T_{80}), hence, the HRT in the CSTR was set to 25 days [16].

The operation and performance parameters of the CSTR are summarized in Table 4, and the experiment lasted for 200 days. In P1 and P2, the daily feeding was CM alone. P1 served as the acclimation period, reflected by the low methane yield (102 mL/g/d) and methane content (52%). Then, the OLR was further increased and after two HRTs, an improved methane yield (146 mL/g/d) and methane content (56%) were obtained in P2. Furthermore, an optimal pH (7.8) and moderate TVFAs/TA ratio (0.26) were found in P2. This suggested that, a stable environment was established and the methane potential of using CM alone was determined. When CM was half replaced by SM in P3, the methane yield increased to 179 mL/g/d, which was consistent with the batch results ($p<0.05$). This enhancement may be due to a more balanced condition favorable for the microbial community in the co-digestion scenario [20]. This was also supported by the lower concentrations of TVFAs and SCOD in P3 (644 mg/L and 3381 mg/L) than in P2 (782 mg/L and 3798 mg/L), implying more intermediate products were converted

Table 3
Functional enzymes and microbial species that produce them (Numbers in last column: 1:CM on day 75, 2:CM:SM = 3:1 on day 75, 3:CM:SM = 1:1 on day 75, 4:CM:SM = 1:3 on day 75, 5:SM on day 75, 9: CM on day 0, 10 SM on day 0).

Key-enzymes	Function	Species	Treatment
Cellulase	Cellulose/hemi-cellulose metabolism	Bacteroides sp.	10
		Uncultured bacteria sp.	10
glutamate decarboxylase	Butanoate metabolism	Barnesiella intestinihominis	10
glycogen phosphorylase	Starch and sucrose metabolism	Clostridium sp. CAG:448	10
alpha-amylase	Starch and sucrose metabolism	bacterium P201	10
		Bacteroides sp. 43_108	10
		Alistipes	9; 10
		Entamoeba dispar SAW760	9; 10
		Entamoeba invadens IP	10
coenzyme-B sulfoethylthiotransferase	Methane metabolism	Methanoculleus sp.	1; 3;4
		Methanoculleus marisnigri	1; 3;4; 5
		Methanoculleus sp. MH98A	1; 2;3; 4
		Methanoculleus thermophilus	1; 2;3; 4;5
		Methanoculleus chikugoensis	2; 3
		Methanoculleus sediminis	4
		Methanolinea tarda	1; 2;5
		Methanobacterium petrolearium	1; 3
		Methanobacterium formicicum	1; 3;5
		Methanobacterium sp.	1; 5
		Methanomassiliicoccus luminyensis	3
		Methanothrix soehngenii	1; 2;3; 4;5
		Methanospirillum hungatei	1; 2;4; 5
		Methanospirillum hungatei JF-1	5
tetrahydromethanopterin S-methyltransferase	Methane metabolism	Methanoculleus marisnigri	3
		Methanoculleus sp. MH98A	4
		Methanobacterium sp. SMA-27	5
		Methanothrix soehngenii	1; 2
methylenetetrahydromethanopterin dehydrogenase	Methane metabolism	Methanoculleus marisnigri	3
		Methanoculleus sediminis	3
formylmethanofuran dehydrogenase	Methane metabolism	Methanosphaerula palustris	2
CoB-CoM heterodisulfide reductase	Methane metabolism	Methanospirillum hungatei	2
		Methanothrix soehngenii GP6	5
		Smithella sp. SCADC	5
coenzyme F420 hydrogenase	Methane metabolism	Methanospirillum hungatei JF-1	3

Table 4
Performance of continuous reactor at mesophilic temperature.

Feed composition (%) VS basis	OLR (g VS/L/Day)	Days	Daily Methane yield (mL/gVS_{added}/d)	Methane content (%)	pH	TVFAs (mg/L)	TA (mg/L)	TVFAs/TA	SCOD (mg/L)
CM-100	0.5	0-50(P1)	102(19)	52(2)	7.6(0.1)	592(60)	2557(312)	0.23(0.01)	ND
CM-100	1	50-100(P2)	146(7)	56(1)	7.8(0.1)	782(58)	3017(112)	0.26(0.01)	3798(66)
CM-50 SM-50	1	100-150(P3)	179(11)	61(1)	7.5(0.1)	644(68)	2573 (77)	0.26(0.02)	3381(193)
CM-100	1	155-200(P4)	159(4)	59(0)	7.6(0.1)	664(22)	2898(126)	0.23(0.01)	3585(158)

Values are expressed as mean and standard deviations between brackets. ND: not determined.

into methane by the newly formed microbial guilds, especially the methanogens. Moreover, co-digestion didn't trigger any inhibition, suggested by a comparable pH (7.5) and TVFAs/TA (0.26) compared with P2 ($P^{*}0.05$) (Table 4). To get rid of the influence of the remaining organic compounds in SM, data was recorded from 155 onwards when feeding back to CM in P4. The daily withdraw-fed mode imposed a strong influence on the microbial community, resulting in the dropping methane yield (159 mL/g/d). However, the microbial synergy remained as the methane yield in P4 was significantly higher than that in P2 (146 mL/g/d), indicating the newly formed methanogenic consortia at the end of P3 were not fully washed out and could still contribute to the methane production ($P^{*}0.05$). The improved methane yield observed in P3 and P4 could be ascribed to a concomitance between acetotrophic and hydrogenotrophic archaeal community, as indicated by Li et al. [19] and Ros et al. [32], this hypothesis was further proved by our microbial data and found more even distribution of archaeal community in P3 and P4 than in P2 (dominated by *Methnosaeta* only). Particularly, genus *Methanoculleus* which was correlated with the enhanced methane yield in the batch tests, was higher in P3 (13%) and P4 (17%) than in P2 (3%) (Fig. S4). Additionally, the profusion of syntrophic bacteria *Candidatus Cloacimonas* was obtained in P3 (20%) and P4 (19%) instead of P2 (6%), which could also benefit the methane production (Fig. S3).

Although batch and continuous experiments performed at lab-scale can only describe a simplified model of a real farm-scale installation, the present study was able to describe synergistic effects derived from the co-digestion of CM and SM on a biochemical and microbiological level. From an application-oriented point of view, for dairy farms where both CM and SM are available, it is possible to improve the volumetric methane production efficiency of the agricultural AD plant (by 20%) through the co-digestion of CM and SM instead of CM alone. Such co-digestion approach also favors the enrichment of the archaeal community, especially hydrogenotrophic archaea, which are essential for an improved methane yield, as shown in our experiments. However, when SM is not sufficiently available, the periodical addition of SM to co-digest with CM is an alternate, generating less but demanding synergy (10%) compared with digesting CM alone. Since the OLR adopted in our test is rather low, future investigation on high-solid input AD based on the co-digestion pattern is required.

4. Conclusions

Co-digestion of CM and SM was investigated in both batch and continuous modes. Lignocellulose content removal and methane yield in the batch test were improved when using a mixture of CM and SM (3:1, 1:1, 1:3 on VS basis) as opposed to CM only. Moreover, co-digestion resulted in synergistic methanogenesis that was 3.4%–10.1% higher than would be predicted from digesting CM or SM alone. Through high-throughput sequencing, microorganisms present in SM contributed to the improved lignocellulose degradation in CM. The co-digestion strategy also resulted in different microbial communities, especially on the enrichment of *Candidatus Cloacimonas* and *Methanoculleus*. Those two genera were responsible for the synergy observed in both batch and continuous modes. Additionally, an improved methane yield (20%) was obtained in the continuous reactor when SM was co-digested with CM (1:1 on VS basis) at an OLR of 1 g/L/d and a HRT of 25 days. Returning to feeding the same reactor with only CM, the methane yield decreased but remained 10% higher than the methane yield in phase P2, indicating that the microbial consortia that were formed in the presence of SM are relatively functional.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Yu Li: Conceptualization, Investigation, Writing - original draft. **Spyridon Achinas:** Methodology. **Jing Zhao:** Resources. **Bert Geurkink:** Methodology, Software. **Janneke Krooneman:** Writing - review & editing. **Gerrit Jan Willem Euverink:** Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.renene.2020.02.041.

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